Available online at www.sciencedirect.com
ScienceDirect
Veterinary Immunology and Immunopathology 124 (2008) 341–354
Veterinary immunology and immunopathology
www.elsevier.com/locate/vetimm

Construction and application of an avian intestinal intraepithelial lymphocyte cDNA microarray (AVIELA) for gene expression profiling during *Eimeria maxima* infection

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Received 12 July 2007; received in revised form 14 April 2008; accepted 22 April 2008

Abstract

Intestinal intraepithelial lymphocytes (IELs) are the primary immune effector cells in the gut and play a critical role in eliciting protective immunity to enteric pathogens such as *Eimeria*, the etiologic agent of avian coccidiosis. In this study, a microarray of genes expressed by intestinal IELs from *Eimeria*-infected chickens was constructed using the expressed sequence tag (EST) strategy. The avian intestinal IEL cDNA microarray (AVIELA) contained duplicates of 9668 individual ESTs (6654 known genes and 3014 unique singletons of unknown identity) and was used to analyze gene expression profiles during primary and secondary *Eimeria maxima* infections. Following primary inoculation with *E. maxima*, the expression levels of 74 genes were significantly altered more than two-fold over the 3-day infection period (51 up-regulated, 23 down-regulated). Following secondary infection, the expression levels of 308 genes were significantly altered (62 up-regulated, 246 down-regulated). Pathway gene analysis indicated that many of the modulated genes were related to apoptosis, JAK/STAT, MAPK, interleukin, and TLR signaling pathways, and involving innate and adaptive immune responses. This chicken IEL microarray will provide a valuable resource for future transcriptional profiling of the genes involved in protective immunity to chicken enteric pathogens.
Published by Elsevier B.V.

Keywords: Intraepithelial lymphocytes; *Eimeria*; cDNA microarray; Coccidiosis; Mucosal immunity; Chicken; Mucosal pathogen

Abbreviations: AVIELA, avian intestinal IEL cDNA microarray; EST, expressed sequence tag; IEL, intraepithelial lymphocyte; MAPK, mitogen activated protein kinase; MLF2, myeloid leukemia factor 2; PPAR, peroxisome proliferator-activated receptor; TLR, Toll-like receptor; TNF, tumor necrosis factor; DUSP, dual specificity MAPK phosphatase.

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0165-2427/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.vetimm.2008.04.013

1. Introduction

Avian coccidiosis is a major parasitic disease of poultry caused by the apicomplexan protozoa *Eimeria*. Coccidiosis seriously impairs the growth and feed utilization of infected birds resulting in loss of productivity in excess of $3 billion annually worldwide (Shirley et al., 2004; Williams, 1999). Conventional disease control strategies rely heavily on chemoprophylaxis and, to a certain extent, immunization with live vaccines. However, increasingly restrictive governmental regulation of anticoccidial drugs and the high costs of new drug/vaccine development have stimulated the need for novel and alternative control strategies against coccidiosis. These new approaches will be realized only after systematic and detailed analysis of host–parasite interactions at the molecular and cellular levels. Towards this end, considerable research efforts have been directed at identifying host protective immune mechanisms during avian coccidiosis (Blake et al., 2006; Dalloul and Lillehoj, 2005; Innes and Vermeulen, 2006; Shirley et al., 2005).

Intestinal mucosal surfaces are defended against enteric pathogens by the gut-associated lymphoid tissues. The primary immune effector cells of the intestinal mucosa are intraepithelial lymphocytes (IELs), localized in the outer epithelial layer, that recognize and destroy pathogens that breach the intestinal epithelium. Chicken intestinal IELs and their cytokines generated during a primary immune response against *Eimeria* protozoa are principally responsible for protective immunity against subsequent secondary infections (Lillehoj and Trout, 1996). We, as well as another group, have previously reported that the number of gut IELs expressing CD3, CD4, and CD8, and the αβ- and γδ-T cell receptor, were significantly increased within 6–8 days following primary experimental infection with *Eimeria* parasites (Hong et al., 2006b; Rothwell et al., 1995). However, the levels of only $CD4^+$ T cells remained elevated following secondary infection suggesting that this subpopulation of intestinal lymphocytes mediates protective immunity against coccidiosis.

The central role of cellular immunity mediated by intestinal IELs in conferring protection against avian coccidiosis has been documented (Lillehoj et al., 2004; McDonald, 1999). However, further scientific progress in this area has been hampered for a variety of reasons, mainly the lack of DNA sequence homology between mammal and avian immune-regulated genes (Staeheli et al., 2001). As a result, identification and cloning of the avian genes have been arduous processes and few cross-reactive monoclonal antibodies or bioassays exist. While the recently announced draft sequence of the chicken genome has led to the identification of many previously unknown genes via *in silico* prediction and expressed sequence tag (EST) clustering methods, the functions of most of those genes remain to be established.

In an attempt to circumvent this problem, a small number of low- and high-density cDNA microarrays incorporating immune and non-immune genes have been developed (Bliss et al., 2005; Burnside et al., 2005; Cogburn et al., 2003; Koskela et al., 2003; Morgan et al., 2001; Neiman et al., 2003; Smith et al., 2006). In addition, a consortium of research groups has developed a comprehensive 13,000 element chicken cDNA microarray (Burnside et al., 2005) and a whole chicken genome oligonucleotide array (Affymetrix Corp., Sunnyvale, CA) is commercially available. Our own studies have focused on gene expression in intestinal IELs using a cDNA library of *Eimeria*-infected chickens (Min et al., 2005). We have now used this library to develop a second-generation, tissue-specific 9.6 K element microarray to investigate transcriptional regulation of the immune response to intestinal pathogens. Our results, reported herein, demonstrated that this array effectively identified modified transcriptional responses following primary or secondary infections with *Eimeria maxima* parasites.

2. Materials and methods

2.1. Microarray construction

The AVIELA was constructed from 9668 EST clones that were selected from previously prepared 14,409 IEL clones after removing redundant sequences. Composition of the array elements is shown in Table 1. The elements were organized into duplicated 24 subarrays, each as a 21×21 spot grid. Prior to spotting, approximately 1.0 μl of each clone was transferred from 384-well plates to 100–150 μl of LB medium containing 100 μg/ml ampicillin in 96-well plates, and incubated overnight at 37 °C. Plasmid DNA was prepared using the REAL Prep 96 kit (Qiagen, Valencia, CA) and cDNA inserts were amplified using PCR SuperMix (Invitrogen, Carlsbad, CA) in 50 μl reactions with 1.0 μl of plasmid DNA as template. Both the initial PCR reactions and PCR purifications were confirmed by agarose gel electrophoresis. Purified PCR products were dried and resuspended in 5.0 μl of 3× SSC (1× = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) containing 0.01% SDS to 150 μg/ml and 1.0 μl was

Table 1
Composition of the AVIELA

Type of cDNA clones	Number of clones	Number of spots	Detail description
IEL ESTs	9668	19,336	6654 genes and 3014 singleton ESTs
Controls	6	76	GAPDH, β-actin, soybean genes, and vectors
Blanks	N/A	494	Spotting solution only
Total number of elements	9674	19,906	

spotted in duplicate (19,690 total spots containing genetic elements) onto Telechem SuperAmine slides (Telechem International, Sunnyvale, CA) using an OmniGrid Accent spotter (GeneMachines, San Carlos, CA). Each subarray contained replicate spots of spotting solution alone as negative controls. The PCR products were fixed to the slides by UV cross-linking at 400 mJ and the slides were blocked in 1% BSA, 0.1% SDS, 2× SSC for 20 min at 55 °C. Spotting quality was evaluated by hybridization of representative slides with SYBR-Green II according to the manufacturer's protocol (Invitrogen).

2.2. Animals, parasites, and experimental infections

Fertilized eggs of White Leghorn SPAFAS chickens (Charles River Laboratories, Storrs, CT) were hatched at the Animal and Natural Resources Institute (Beltsville, MD). Animals were given free access to feed and water, and constant light was provided during the entire experimental period. As described previously, wild-type strain 41-A *E. maxima* oocysts were cleaned by flotation on 5.25% sodium hypochlorite and washed 3 times with sterile PBS (Hong et al., 2006b). Ninety-six chickens were evenly divided into 8 groups (12 chickens/group) for primary or secondary infections with *E. maxima*. For primary infection, 3 groups of chickens at 3 weeks of age were orally inoculated with 1.0×10^4 sporulated oocysts/chicken. Non-infected 3-week-old chickens were used as primary day 0 negative controls. For secondary infection, 3 groups received the same primary inoculation followed by oral infection at 6 weeks of age with 2.0×10^4 sporulated oocysts. Non-infected 6-week-old chickens were used as secondary day 0 control. Our prior studies have documented day 0 non-infected controls, rather than age-matched controls, for comparison with infected groups (Hong et al., 2006a,b; Min et al., 2003, 2005). Moreover, no significant transcriptional differences were apparent when we compared the gene expression patterns of IELs between two different age groups of birds given primary and secondary infections.

2.3. Isolation of intestinal IELs, RNA preparation, and microarray hybridization

Intestinal IELs were prepared as previously described (Hong et al., 2006b). At 0, 1, 2, and 3 days following primary or secondary infections, the intestinal jejunum was removed from 12 chickens in each group, cut longitudinally, and washed three times with ice-cold Hank's balanced salt solution (HBSS) containing 100 μg/ml of penicillin and 100 mg/ml of streptomycin (Sigma, St. Louis, MO). The mucosal layer was carefully removed using a surgical scalpel, the tissue was washed several times with HBSS containing 0.5 mM EDTA and 5% fetal calf serum (FCS) and incubated for 20 min at 37 °C with constant swirling. Cells released into the supernatant were pooled, passed through nylon wool (Robbins Scientific, Sunnyvale, CA) to remove dead cells and cell aggregates and washed twice with HBSS. IELs were purified on a discontinuous Percoll density gradient by centrifugation at 600 g for 25 min at 24 °C. Harvested IELs were adjusted to $1–2 \times 10^6$/ml and their purity as determined by flow cytometry was 80–90%. All experiments were approved by the Animal and Natural Resources Institute IACUC.

IELs from each group of 12 chickens were pooled, total RNA was prepared using TRIzol (Invitrogen) and the RNeasy Mini RNA Purification Kit (Qiagen), and aminoallyl-labeled RNA was prepared using the Amino Allyl Message Amp II aRNA Amplification Kit according to the manufacturer's protocol (Ambion, Austin, TX). Briefly, first strand cDNA was prepared by reverse transcription from 2.0 μg of RNA using a modified oligo-dT primer containing a T7 RNA polymerase binding site on the 5′ end, followed by second strand cDNA synthesis. The double-stranded cDNA was transcribed to aminoallyl-labeled RNA using T7 RNA polymerase with aminoallyl-UTP. Two 20 μg aliquots of each aminoallyl-RNA sample were fluorescently labeled with AlexaFluor 555 or AlexaFluor 647 (Invitrogen) according to the manufacturer's instructions and labeled RNAs were column-purified using the RNA

Amplification Kit (Ambion). Concentration and labeling efficiencies of RNAs were determined spectrophotometrically. A circular loop design was employed for the hybridizations (day 0 vs. day 1, day 1 vs. day 2, day 2 vs. day 3, and day 3 vs. day 0) (Bliss et al., 2005; Townsend, 2003). Two-color microarray hybridizations were performed using HybIt hybridization buffer (Telechem) in Mica hybridization chambers (GeneMachines) at 50 °C overnight. After hybridization, the slides were rinsed in 0.5× SSC, 0.01% SDS at room temperature and washed once for 15 min in 0.2× SSC, 0.2% SDS at 50 °C, 3 times for 1 min in 0.2× SSC at room temperature, and 3 times for 1 min in distilled water at room temperature and scanned with an ArrayWoRx scanner (Applied Precision, Issaquah, WA) using appropriate filters.

2.4. Microarray data acquisition, processing, and analysis

Microarray data acquisition, processing, and analysis were performed as described previously (Dalloul et al., 2007). Spot and background intensities were acquired using SoftWoRx tracker (Applied Precision) and data analysis was performed using GeneSpring version 7.3 (Silicon Genetics, Redwood City, CA). For each spot, the mean intensity was obtained by subtraction of the mean background intensity from the mean foreground intensity. Quality control of individual probes was performed to remove anomalous spots (dust, air bubbles) and low signal spots. The criterion for spot acceptance required an intensity which was greater than mean background intensity plus 2 standard deviation of mean background intensity. Spot intensities from each channel on each slide were normalized to the total spot intensity of each channel and fold change and statistical analyses were performed in GeneSpring. High quality spot data containing at least one replicate were subjected to fold change analysis. Elements exhibiting greater than 2.0-fold changes in expression in at least one of the 3 time points in each infection were selected and applied to the one-way ANOVA test ($P < 0.05$). This list of elements was used for generating one time point profiles as well as query genes for pathway searching.

2.5. Bioinformatic analysis

All sequence data files were obtained from NCBI (http://www.ncbi.nlm.nih.gov). IEL cDNA elements used to create the AVIELA were mapped to the chicken genome reference assembly (version 2.1) and reference RNA and protein sequences (formatted database for Blast, May 2006) using NCBI Blast (version 2.2.13). The criteria for the acceptance of Blast search results were alignment length $\geq$100 nucleotides and *e*-value $\leq 1e^{-100}$ for DNA sequences and alignment length $\geq$30 amino acids and *e*-value $\leq 1e^{-10}$ for protein sequences. Entrez gene data and Homologene data (October 2006) from NCBI were used to gather gene information (identification number, symbol, and name). Geneontology (GO) annotations were extracted from gene to GO data in NCBI. To analysis pathway information, chicken Entrez gene IDs were mapped to Homologene IDs (locus link IDs) for human, which were used for analyses of pathway classification from the PANTHER database (http://www.pantherdb.org). Python (version 2.2.4) and SQL scripts were used to process and manipulate Blast results and data processing, respectively. Data for genes from the chicken genome were obtained from Ensembl database release 42 (http://www.ensembl.org).

2.6. Quantitative RT-PCR

To confirm gene expression changes observed by microarray analysis, quantitative RT-PCR was performed as described (Hong et al., 2006a). Eight genes were selected based on the AVIELA results (CD74, FABP1, IL1R, JAK, JUN, MLF2, RIPK1, and STAT5B), and their mRNAs levels were quantified and normalized to GAPDH mRNA levels using gene-specific forward and reverse primers (supplement 1). Amplification and detection were carried out using equivalent amounts of the same IEL RNA samples used for microarray hybridizations with the Mx3000P system and Brilliant SYBR Green QRT-PCR master mix (Stratagene, La Jolla, CA). Standard curves were generated using the Q-gene program (Muller et al., 2002). Each experiment was performed in triplicate and the two-fold diluted standard RNA was used to generate standard curves. To normalize RNA levels between samples within an experiment, the mean threshold cycle (C_t) values were calculated by pooling values from all samples in that experiment. Transcript levels were normalized to those of GAPDH using the Q-gene program.

3. Results

3.1. Characterization of the AVIELA

The AVIELA contained 9668 ESTs that were selected from our previously reported 14,409 EST clones from a chicken intestinal IEL library after eliminating the most redundant sequences (Min et al., 2005). Of these, 6654

ESTs (69%) were identified as known genes and 3014 (31%) were unique singletons of unknown identity by Blast search (Table 2). In addition, 8540 ESTs (86.7%) were mapped on the chicken reference genome assembly producing 43.5% coverage of the entire genome. Coverage on individual chromosomes ranged from 30.8% (mitochondrial chromosome) to 53.9% (chromosome 19). Of the 1128 ESTs that could not be assigned to assembled chromosomes, 649 were unmapped genes and 479 were unmapped singletons. All elements were spotted in duplicate, to improve the statistical power and reproducibility of the array, creating an AVIELA containing 19,906 individual spots, including controls and blanks (Table 1). Spotting quality and size were verified by staining of representative slides with SYBR-Green II according to the manufacturer's protocol (Invitrogen). Greater than 90% of the spots possessed an average diameter of 150–200 μm.

3.2. IEL EST expression profiling during Eimeria infections

The AVIELA array was applied to profile transcriptional changes following primary and secondary *E.*

Table 2
Chromosomal distribution of genes and singleton ESTs on the AVIELA

Chromosome number	*N* of genes on chromosome[a]	*N* of genes on the array[b]	*N* of singleton ESTs on the array[c]
1	2,177	814 (37.4%)	351
2	1,432	525 (36.7%)	235
3	1,266	450 (35.5%)	239
4	1,144	407 (35.6%)	209
5	969	392 (40.5%)	182
6	571	214 (37.5%)	87
7	536	208 (38.8%)	86
8	544	220 (40.4%)	84
9	461	187 (40.6%)	103
10	436	165 (37.8%)	61
11	396	159 (40.2%)	58
12	339	133 (39.2%)	60
13	352	148 (42%)	72
14	448	177 (39.5%)	80
15	393	203 (51.7%)	48
16	56	26 (46.4%)	4
17	311	126 (40.5%)	46
18	342	120 (35.1%)	48
19	321	173 (53.9%)	48
20	361	146 (40.4%)	55
21	251	119 (47.4%)	38
22	122	53 (43.4%)	17
23	235	116 (49.4%)	44
24	196	70 (35.7%)	25
25	128	46 (35.9%)	11
26	260	125 (48.1%)	53
27	277	100 (36.1%)	16
28	215	114 (53%)	31
32	0	0	0
MT	13	4 (30.8%)	10
W	2	1 (50%)	0
Z	730	264 (36.2%)	134
UM[d]	N/A[e]	649	479
Total	15,284	6654 (43.5%)	3014

[a] Number (*N*) of genes on chromosome indicates the sum of the "known protein-coding genes" and "novel protein-coding genes" provided by Ensembl release 42 (chicken genome ver 2.1).
[b] Percentages were calculated by dividing the number of genes on the array by the number of genes on the chromosome from Ensembl.
[c] Singleton ESTs constitute sequences in the genome and on the array, but not matched with known genes on the chromosome.
[d] UM, unmapped genes or ESTs.
[e] N/A, not applicable.

maxima infections as described in Section 2. By image and data analyses of microarray images and spot data, it was determined that 5872 elements on the AVIELA contained at least one replicate spot of high quality data, which were used for all subsequent analyses to avoid generating incomplete expression profiles. To determine chicken intestinal IEL RNA expression levels, the one-way ANOVA test was used to compare RNA levels between days 1, 2, and 3 post-primary or post-secondary infections, and the transcripts that showed more than two-fold up- or down-regulation at least once over the 3-day period were selected. As shown in Table 3, following primary *E. maxima* infection, 74 transcripts were significantly changed ($P < 0.05$) (51 increased, 23 decreased). Following secondary infection, 308 mRNAs were significantly modulated (62 increased, 246 decreased). Interestingly, gene expression tended to be suppressed during secondary infection compared with primary infection, and most of the suppression was observed at day 3 post-secondary infection (data not shown). The 20 transcripts exhibiting the greatest levels of up- or down-regulation during primary or secondary infection are listed in Tables 4 and 5 with their Geneontology process information. Only 3 transcripts were commonly modulated during both primary and secondary infections, 2 unidentified ESTs being up-regulated (GenBank accession numbers CD734414 and CD734999) and 1 mapped gene being down-regulated (etoposide-induced 2.4, EI24). CD734414 and CD734999 were induced during primary infection, but suppressed during secondary infection (Table 4, additional supplements 2 and 3), while EI24 was decreased in both primary and secondary infections (Table 5, additional supplements 2 and 3). A complete list for all the transcripts altered more than two-fold is available in the supplement.

3.3. Gene function analyses

Geneontology (GO) function analyses of the genes significantly modulated >two-fold during the course of primary or secondary *E. maxima* infections were performed using GO annotation and GO slim information from NCBI. As shown in Fig. 1A, biological process analysis showed that the largest groupings of modulated genes were related to metabolism, including macromolecule metabolism, cellular physiological processes, and transport. Interestingly, genes related to biosynthesis were highly induced following primary *E. maxima* infection. Conversely, the induction of genes related to nucleotide and nucleic acid metabolism, regulation of biological processes, and cell communication mainly occurred following secondary *E. maxima* infection. Similarly, molecular function analysis of these genes revealed that the most prominent functions were related to binding (including nucleic acid and protein binding) and transferase, hydrolase, ligase, and transporter activities (Fig. 1B). The genes related to isomerase and structural molecule activities were more prominently up-regulated following primary infection, whereas genes with oxidoreductase activity were up-regulated after secondary infection.

Finally, we specifically focused our investigation on gene relevant to immune-related pathways. For this analysis, all two-fold altered genes were used, regardless or statistical significance, to gather as much information as possible on immune-related pathways, as suggested in a recent report (Guo et al., 2006). As

Table 3
Global summary of the AVIELA analysis and differentially modulated elements in primary or secondary *E. maxima* infections

Expression description	*N* of elements
Total number of elements on the IEL array	9,668
Elements with at least one high-quality replicate spot on each slide	5,872
More than 2-fold change with statistically significant ($P < 0.05$)[a]	
Primary *E. maxima* infection	74
Elements exhibiting increased expression	51
Elements exhibiting decreased expression	23
Secondary *E. maxima* infection	308
Elements exhibiting increased expression	62
Elements exhibiting decreased expression	246
Primary and secondary *E. maxima* infections (common)	3
Elements exhibiting increased expression in primary infection	2
Elements exhibiting decreased expression in primary infection	1
Elements exhibiting increased expression in secondary infection	0
Elements exhibiting decreased expression in secondary infection	3

[a] Statistical significance of two-fold changed elements was derived by 1-way ANOVA test ($P < 0.05$) described in Section 2.

Table 4
The 20 most up-regulated elements and GO biological terms of the AVIELA with ≥ significant two-fold change ($P < 0.05$) at any day following primary or secondary *E. maxima* infection

GBID[a]	Fold change	*P* value	Gene symbol	Description	GO process
Primary *E. maxima* infection					
CD734414	18	0.026			
CD730632	17	0.013	LOC490735	Similar to farnesyltransferase, CAAX box, beta	
CD735670	16	0.017	CIAPIN1	Cytokine-induced apoptosis inhibitor 1	Anti-apoptosis
CD739293	13	0.013			
CD731397	12	0.002	LOC419752	Similar to guanine nucleotide exchange factor	
CD732440	11	0.034	LOC417378	Similar to Casein kinase 1, delta	
CD728589	10	0.027	LOC419834	Similar to Kruppel-like transcription factor	Transcription
CD735000	8	0.010	LOC431580	Similar to Ran binding protein 11	
CF075001	5	0.035	RPL17	Ribosomal protein L17	Translation
CD729037	5	0.050	CPM	Carboxypeptidase M	Proteolysis
CF075063	5	0.033	LOC769935	Similar to forkhead box P2	
CD739746	4	0.044			
CD732415	3	0.042	ZWILCH	Zwilch, kinetochore associated, homolog	
CD729887	3	0.022	NSMCE1	Non-SMC element 1 homolog (*S. cerevisiae*)	DNA repair
CD729850	3	0.001	ALDOB	Aldolase B, fructose-bisphosphate	Glycolysis; fructose metabolism
CD732872	3	0.010	RPL18A	Ribosomal protein L18a	Translation
CD739377	3	0.009	OBFC2A	Oligonucleotide/oligosaccharide-binding fold containing 2A	
CD728204	3	0.026	LGALS3	Lectin, galactoside-binding, soluble, 3	Skeletal development
CD731744	3	0.003	PGDS	Prostaglandin-D synthase	Prostaglandin biosynthesis; lipid metabolism
CD728315	3	0.010	LOC693257	NK-lysin	Lipid metabolic process; defense
Secondary *E. maxima* infection					
CD727518	9	0.050			
CD737831	8	0.017	WIPI2	WD repeat domain, phosphoinositide interacting 2	
CD734811	7	0.041	PSEN1	Presenilin 1	Apoptosis
CD729201	7	0.007			
CD727013	6	0.050			
CD727819	4	0.019	LOC420714	Similar to mKIAA1042 protein	
CD736320	4	0.050	LPIN2	Lipin 2	Metabolic process; transport
CD728258	3	0.032	TMEM41B	Transmembrane protein 41B	
CD737594	3	0.010			
CD736710	3	0.029	LOC423106	Similar to Efcab4a protein	Small GTPase mediated signal transduction
CD734213	3	0.001	NIPA2	Non-imprinted in Prader-Willi/ Angelman syndrome 2	
CF075102	3	0.014	DUSP1	Dual specificity phosphatase 1	Cell cycle; response to oxidative stress;
CD727801	3	0.000	BAP1	BRCA1 associated protein-1 (ubiquitin carboxy-terminal hydrolase)	Ubiquitin-dependent protein catabolism; negative regulation of cell proliferation
CD727457	3	0.003	LOC427365	Similar to Phospholipase A-2-activating protein (PLAP)	
CD737216	3	0.045			
CD730076	3	0.007	BRD1	Bromodomain containing 1	
CD731812	3	0.042	LOC420252	Similar to KIAA0896 protein	
CD727585	2	0.018	CA5B	Carbonic anhydrase VB, mitochondrial	One-carbon compound metabolism
CD736739	2	0.008	SLC27A4	Solute carrier family 27 (fatty acid transporter), member 4	Lipid metabolic process; transport
CD737331	2	0.048			

[a] GBID; GenBank accession ID, blank indicates unidentified EST (singleton) for gene symbol and description or no mapped data for the GO process.

Table 5
The 20 most down-regulated elements and GO biological terms of the AVIELA with ≤ significant two-fold change ($P < 0.05$) at any day following primary or secondary *E. maxima* infection

GBID[a]	Fold change	*P* value	Gene symbol	Description	GO process
Primary *E. maxima* infection					
CD730488	−61	0.037	LOC424042	Similar to MGC81019 protein	
CD737238	−16	0.000	LOC417569	Similar to MGC80576 protein	
CD739377	−11	0.009	OBFC2A	Oligonucleotide/oligosaccharide-binding fold containing 2A	
CD728051	−8	0.036	DNAL4	Dynein, axonemal, light chain 4	Microtubule-based movement
CD728681	−7	0.020	DNAJA1	DnaJ (Hsp40) homolog, subfamily A, member 1	Protein folding
CD728086	−6	0.030			
CD734333	−6	0.025			
CD739791	−6	0.041	STAT3	Signal transducer and activator of transcription 3	Cytokine and chemokine mediated signaling pathway; JAK-STAT cascade
CD729342	−5	0.000	LOC416154	Similar to intestinal 15 kda protein; I-15P	
CD728204	−5	0.026	LGALS3	Lectin, galactoside-binding, soluble, 3	
CD735495	−3	0.040	HMG-17	HMG-17 protein	
CD731378	−3	0.008	EI24	Etoposide-induced 2.4	Induction of apoptosis
CD738720	−3	0.003	NDRG1	*N*-myc downstream regulated gene 1	Cell differentiation; response to metal ion
CD735253	−3	0.028			
CD732877	−3	0.026			
CD726937	−3	0.023			
CD729306	−3	0.000	MYST2	MYST histone acetyltransferase 2	Transcription
CD739845	−3	0.006	EXOSC2	Exosome component 2	rRNA processing
CD734619	−2	0.031	TAF15	TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor	Transcription
CF075130	−2	0.018	HSC70	Heat shock cognate 70	Protein folding
Secondary *E. maxima* infection					
CD740204	−100	0.009	LOC416632	Similar to KIAA0350 protein	
CD737331	−23	0.048			
CD735479	−21	0.041	SLC35C2	Solute carrier family 35, member C2	Transport
CD731769	−18	0.001			
CD738344	−16	0.021	PTPLAD1	Protein tyrosine phosphatase-like A domain containing 1	IκB kinase/NF-κB cascade; activation of JNK activity
CD731155	−14	0.001	LOC430480	Hypothetical LOC430480	
CD737216	−13	0.045			
CF075077	−12	0.000	RPS6	Ribosomal protein S6	Translation
CD737714	−11	0.029	RAD21	RAD21 homolog (*S. pombe*)	Cell division
CD730112	−11	0.020	ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	Energy metabolism
CD735959	−10	0.005	WDTC1	WD and tetratricopeptide repeats 1	
CD730076	−10	0.007	BRD1	Bromodomain containing 1	
CD738568	−10	0.011	HDHD4	*N*-Acetylneuraminic acid phosphatase	
CD728026	−9	0.000	NIPA2	Non-imprinted in Prader-Willi/Angelman syndrome 2	
CD729707	−9	0.000	LOC424408	Hypothetical LOC424408	
CD727081	−8	0.034	CSTF2	Cleavage stimulation factor, 3′ pre-RNA, subunit 2, 64 kDa	mRNA polyadenylation; mRNA cleavage
CD734111	−8	0.000			
CF075078	−8	0.000	RCJMB04_7g5	Similar to 60 kDa heat shock protein, mitochondrial precursor (Hsp60)	Protein folding
CD729683	−7	0.000	VPS13D	Vacuolar protein sorting 13 homolog D (*S. cerevisiae*)	Protein localization
CD732119	−7	0.041	ALDH3A2	Aldehyde dehydrogenase 3 family, member A2	Lipid metabolism

[a] GBID; GenBank accession ID, blank indicates unidentified EST (singleton) for gene symbol and description or no mapped data for GO process.

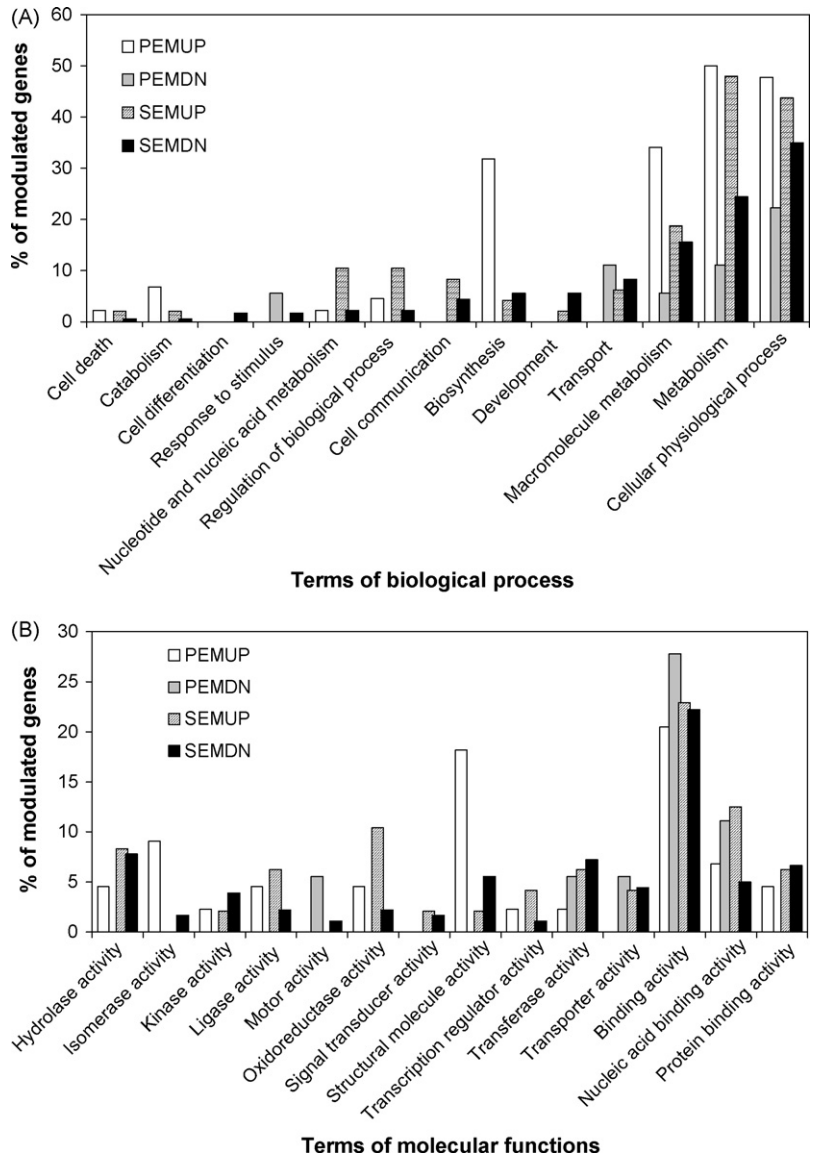

Fig. 1. Distribution of GO annotations of biological processes terms (A) and molecular functions terms (B) of the known genes on the AVIELA. PEM, primary *E. maxima* infection; SEM, secondary *E. maxima* infection; UP, up-regulated genes; DN, down-regulated genes.

shown in Table 6, many of these modulated genes were related to apoptosis, JAK/STAT signaling, MAPK signaling, PPAR signaling, and the ubiquitin/proteosome system.

3.4. Validation of gene expression by quantitative RT-PCR

Expression patterns observed from microarray analysis were validated by quantitative RT-PCR with 8 selected immune-related genes (Table 6). These genes included 4 whose expression was significantly modulated following *Eimeria* infection (FABP1, JAK, JUN, and STAT5B) and 4 non-significantly modulated genes (CD74, IL-1R, MLF2, and RIPK1). As with the AVIELA analysis, the FABP1, JAK, JUN, and STAT5B genes were found to be significantly differentially expressed ($P < 0.05$) when comparing day 1, 2, or 3 post-primary or secondary infection with the respective controls, although FABP1 expression determined by RT-PCR was higher than that observed from the microarray data. Similarly, CD74, IL-1R, MLF2, and RIPK1 showed similar expression patterns when comparing the microarray and quantitative RT-PCR

Table 6
Immune-related genes from pathway analysis with more than 2-fold altered expression in primary or secondary *E. maxima* infection.

Pathway name	GBID[a]	Gene name and symbol	Primary *E. maxima* infection[b]			Secondary *E. maxima* infection[b]		
			1 DPI	2 DPI	3 DPI	1 DPI	2 DPI	3 DPI
Antigen presentation	CD728688	MHC class I beta-2-microglobulin (B2M)		2.1				
	DN829822	MHC class II-associated invariant chain (CD74)						−2.1 (−1.9**)
Apoptosis	CD733882	B cell translocation gene 1 (BTG1)					−3.0	−8.1
	CD731377	Electron-transfer-flavoprotein, alpha polypeptide (ETFA)					5.6	2.2
	CD730880	Interferon-induced 35 kDa protein (IFI35)		−2.1				
	CD730507	Lamin B2 (LMNB2)						−2.5
	CF074950	Receptor-interacting serine/threonine kinase 1 (RIPK1)			2.7 (1.4)			
	CF074840	Tumor necrosis factor receptor superfamily member 1B (TNFRSF1B)				2.3*		−10.8
	CF074793	TNF receptor superfamily, member 6 (TNFRSF6, Fas)		2.0*				
Interleukin signaling	CD738039	Forkhead box O1A (FOXO1A)			−2.3	−3.0		
	CF075063	Forkhead box P2 (FOXP2)		4.5*	3.4*			
	CD732138	Interleukin 1 receptor (IL-1R)				−2.1 (−2.0*)		
JAK/STAT signaling	CD734480	Janus tyrosine kinase (JAK)				2.2** (2.3**)		−2.8* (−1.7**)
	CD729244	Myeloid leukemia factor 2 (MLF2)	2.3 (1.4**)			2.8 (1.4**)		−2.6 (−2.9*)
	CD739791	Signal transducer and activator of transcription 3 (STAT3)	−5.0**	-5.7**				
	CF074973	STAT4						−4.8**
	CF074788	STAT5B				2.2* (2.2*)		
MAPK signaling	CF075102	Dual specificity phosphatase 1 (DUSP1)				2.9*		−2.1*
	CD740469	Jun oncogene (JUN)				2.4* (1.5*)		
	CD739481	Mitogen-activated protein kinase kinase kinase 1 (MAP3K1)					−2.2*	
	CD734109	Mitogen-activated protein kinase kinase kinase 7 (MAP3K7)		−2.4	−2.6			
	CD736603	Mitogen-activated protein kinase 6 (MAPK6)	2.2			−2.1		
PPAR	CD728264	Acyl-coenzyme A oxidase 1, palmitoyl (ACOX1)				2.1*		
	CD730554	Apolipoprotein A-I (APOA1)						−2.1*
	CD735219	Fatty acid binding protein 1, liver (FABP1)			2.0** (7.8*)			−4.0* (−2.9*)
	CD739995	Nuclear receptor co-repressor 2 (NCOR2)						−2.0

Table 6 (*Continued*)

Pathway name	GBID[a]	Gene name and symbol	Primary *E. maxima* infection[b]			Secondary *E. maxima* infection[b]		
			1 DPI	2 DPI	3 DPI	1 DPI	2 DPI	3 DPI
	CD734069	Nuclear receptor interacting protein 1 (NRIP1)						−2.0
Toll-like receptor signaling	CD731155	Inhibitor of kappa light polypeptide gene (IKBKE)						−13.7*
	CD734383	Interferon regulatory factor 4 (IRF4)				−2.1		
	CD727085	Toll interacting protein (TOLLIP)						−3.2*
Ubiquitin/proteasome	CD731812	E3 ubiquitin protein ligase, HECT domain containing 1 (EDD1)				2.7**		−3.7
	CD732269	Proteasome 26S subunit, ATPase, 2 (PSMC2)						−2.3*
	CD733508	Proteasome 26S subunit, non-ATPase, 3 (PSMD3)						−21.2
	CD727801	Ubiquitin carboxy-terminal hydrolase (BAP1)				2.8	2.4	−2.2*
	CD731359	Ubiquitin-conjugating enzyme E2H (UBE2H)						−2.5**

[a] GBID, GenBank accession ID; –, down-regulated elements; DPI, days post-infection; $*P < 0.05$, $**P < 0.001$.

[b] The value in parentheses indicates fold change from quantitative RT-PCR validation. Fold changes were calculated from mean of target value at a given day/mean of 0 day control as described in Section 2.

data, although the latter method revealed that the fold changes of CD74, IL-1R, and MLF2 were statistically significant.

4. Discussion

This report describes the construction of a chicken IEL microarray and its application to study host gene regulation during experimental avian coccidiosis. Following primary infection with *E. maxima*, the expression levels of 74 elements were significantly up- or down-regulated over the 3-day infection period compared with non-infected controls. Following secondary infection, the expression levels of 308 elements were modulated, but only 3 of these were common to the genes identified during the primary infection. Most of the down-regulated elements occurred at day 3 post-secondary infection. This pattern of gene suppression was also observed at days 3–4 following secondary infection with another parasite, *E. acervulina* (unpublished observations). We speculate that this down-regulation might be a common response during secondary *Eimeria* infections that is associated with reprogramming gene expression during the memory immune response.

E. maxima, *E. acervulina*, *and E. tenella* are the common causative pathogens of avian coccidiosis, one of the most costly endemic diseases to the poultry industry worldwide. In particular, *E. maxima* is recognized as the most immunogenic of these three pathogens, although infection by all three *Eimeria* spp. induces protective immunity against subsequent challenge with the homologous parasite (Fitz-Coy, 1992; Martin et al., 1997). However, protective immunity generated during a primary infection by one *Eimeria* spp. only elicits partial cross-protection against heterologous spp. Further complicating the development of an effective coccidiosis vaccine is the immunological variability that exists among different field strains of the same parasite species (Prowse, 1991; Uchida et al., 1994).

Therefore, one of the goals of the current study was to analyze the dynamics of expression of genes involved in immune-related pathways during experimental coccidiosis to further characterize the nature of host protective immunity. In this regard, we found that many of the modulated genes were related to apoptosis (7 genes), the JAK/STAT pathway (5 genes), MAPK signaling (5 genes), PPAR (5 genes), the ubiquitin/proteosome system (5 genes), interleukins (2 genes), and TLR signaling (2 genes). Following primary infection, the expression of these genes did not show

any distinctive patterns over the 3-day experimental period. Following secondary infection, however, these genes, for the most part, were up-regulated at day 1 post-infection and down-regulated at day 3 post-infection. These findings suggest a more robust recognition of *E. maxima* parasites and induction of protective immunity during the secondary response compared with primary infection.

Previous studies in mammalian systems have documented the function of many of these genes and their encoded gene products. Fas is a cell surface receptor belonging to the TNFR family that induces apoptosis through caspase-mediated signal transduction following binding to the Fas ligand. RIPK1 plays an important role in determining TNF-α-induced apoptosis and NF-κB activation during inflammation (Chan et al., 2003; Li et al., 2006). An intracellular JAK/STAT signaling pathway is activated by multiple cytokines and is involved in various cellular functions, including immunity, apoptosis, and cell growth. Chicken JAK has been suggested to be a homolog of mammalian JAK3, which is involved in signal transduction via the type 1 cytokine receptor family, such as IL-2R, IL-4R, and IL-15R as well as in normal development of T and B cells (Kisseleva et al., 2002; Nosaka et al., 1995; Sofer et al., 1998). Among the STAT family members, STAT3 plays a critical role in cellular immune responses involving IL-6 and acute phase reactants, as well as IL-10, an anti-inflammatory cytokine that inhibits natural killer (NK) cell activity. While the function of MLF2 remains to be clarified, a search of the IntAct protein–protein interaction database (http://www.ebi.ac.uk/intact) revealed that it interacts with STAT3 and is involved in interleukin signaling (Kerrien et al., 2007). Finally, STAT5B is activated by various cytokines, including IL-7, resulting in the production of precursor and mature B cells (Kisseleva et al., 2002).

MAPK pathways regulate apoptosis, immunity, and cell growth and differentiation in response to a myriad of external stimuli, primarily growth factors, cytokines, and chemokines. Three major protein kinases of the MAPK pathway are the extracellular-signal-regulated kinases 1 and 2 (ERK1/2), the JUN N-terminal kinase (JNK), and p38. ERK1/2 are preferentially activated by growth factors, whereas JNK and p38 are preferentially activated by stress and inflammatory cytokines (Liu et al., 2007). Another member that was modulated during *Eimeria* infection, MAP3K7, is required for the activation of NF-κB via formation of a kinase complex with TRAF6, and also the activation of JNK. MAPK6, also known as ERK3, is activated by the dual specificity MAPK phosphatase (DUSP), which promotes the export of TNF-α mRNA from the nucleus to the cytoplasm during apoptosis (Jeffrey et al., 2007). In addition, DUSP1 negatively regulates p38 and JNK which result in attenuating TNF-α and IL-6 production, thereby contributing anti-inflammatory effects (Salojin et al., 2006). Finally, JUN is a transcription factor inducing the expression of proinflammatory cytokine genes through p38 and JNK, while MAP3K1 is positive signal mediator in MAPK signaling (Lang et al., 2006).

TLRs and IL receptors play a critical role in host innate and adaptive immune response to microbial infections (Dinarello, 1998; Iwasaki and Medzhitov, 2004; Medzhitov, 2001). The type of immune responses controlled by these receptors is determined by different downstream pathways, such as the MAPK, JAK-STAT, and NF-κB pathways (Akira and Takeda, 2004). In this study, all of the interleukin-related (IL-1R and FOXO1A) and TLR-related (IRF4, TOLLIP, and IKBKE) genes modulated during experimental coccidiosis were down-regulated during secondary infection. IL-1R recognizes IL1A and IL1B, mediating many immune and inflammatory responses. IRF4 negatively regulates immunity and proinflammatory cytokine production through competition with IRF5 in MyD88-dependent TLR signaling (Negishi et al., 2005). TOLLIP inhibits activation of IRAKs, protein kinases downstream of TLRs, leading to suppression of NF-κB-dependent proinflammatory gene expression (Iwasaki and Medzhitov, 2004). By contrast, IKBKE induces costimulatory and chemotactic molecules involved in T cell stimulation and recruitment through TLR-mediated NF-κB activation (Akira and Takeda, 2004).

Our microarray results were validated by quantitative RT-PCR, which showed similar patterns of gene expression for 8 selected genes, although the absolute values for altered gene expression were not always equivalent by the two methods. These differences might be the result of the normalization methods used. Usually, statistical methods such as RMA or LOWESS are used for microarray data normalization, while the levels of housekeeping genes, commonly GAPDH, are used for quantitative RT-PCR normalization. The latter, however, has been argued to not always accurately provide a negative comparison control (Lee et al., 2002). Alternatively, the detection methods used by the two techniques rely on different nucleic acid binding properties, Cy3 or Cy5 dye binding to aminoallyl-modified RNA during microarray hybridization vs. the SYBR green dye that binds

uniformly to double-strand DNA during real-time RT-PCR.

The current investigation extends our previous study utilizing a first-generation chicken EST microarray containing 400 unique genes to investigate transcriptional changes of cytokine genes in intestinal IELs during infection by *E. maxima* or *E. acervulina* (Min et al., 2003). Although different regions of the small intestine were infected by the two parasites (the jejunum/ileum by *E. maxima* and the duodenum by *E. acervulina*), similar changes in the levels of 6 interleukin mRNAs (IL-1B, IL-2, IL-6, IL-8, IL-15, and IL-18) were observed following infection by either *Eimeria* species. Interestingly, however, the present study did not show significant alteration of any cytokine or chemokine genes included in the AVIELA array (IFN-γ, IL-15, IL-16, IL-18, CCL4, CCL20, Ah221, and K203). This could be due to the different methodology including the different genetic lines of chickens, different microarray platforms, and different data analysis methods used in these two studies, and this information is available in supplement 4. Recently, our study demonstrated that the differences in the MHC genes have a profound effect on the kinetic response of cytokines following *E. maxima* infection (Kim et al., 2008). A second-generation AVELA is currently under development to enhance the analysis of an expanded panel of immune-related genes, including those analyzed in the study by Min et al. (2003).

In summary, this report provides the first comprehensive analysis of chicken intestinal IEL gene expression following infection with viable parasites. The results illustrate the utility of the AVIELA as a tool for elucidating the genetic mechanisms used by immunocytes in intestinal immune responses. While several chicken cDNA microarrays have been described in the literature, the AVIELA represents the first array derived from activated mucosal immune cells. Therefore, we anticipate that this microarray will provide valuable information for further characterization of host protective immunity to chicken enteric pathogens.

Acknowledgements

Author thank Dr. Rami Dalloul, Ms. Margie Nichols, and Ms. Diane Hawkins-Cooper for experimental support. This work was supported by funds from the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, grants # 2002-35204-12368 and # 2004-35204-14798.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetimm.2008.04.013.

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